Fig. 1 a

Fig. 1 b

Fig. 1: Model of a complete IgG molecule and Fab fragment; C constant, V variable, CDR complemenatarity determining regions,

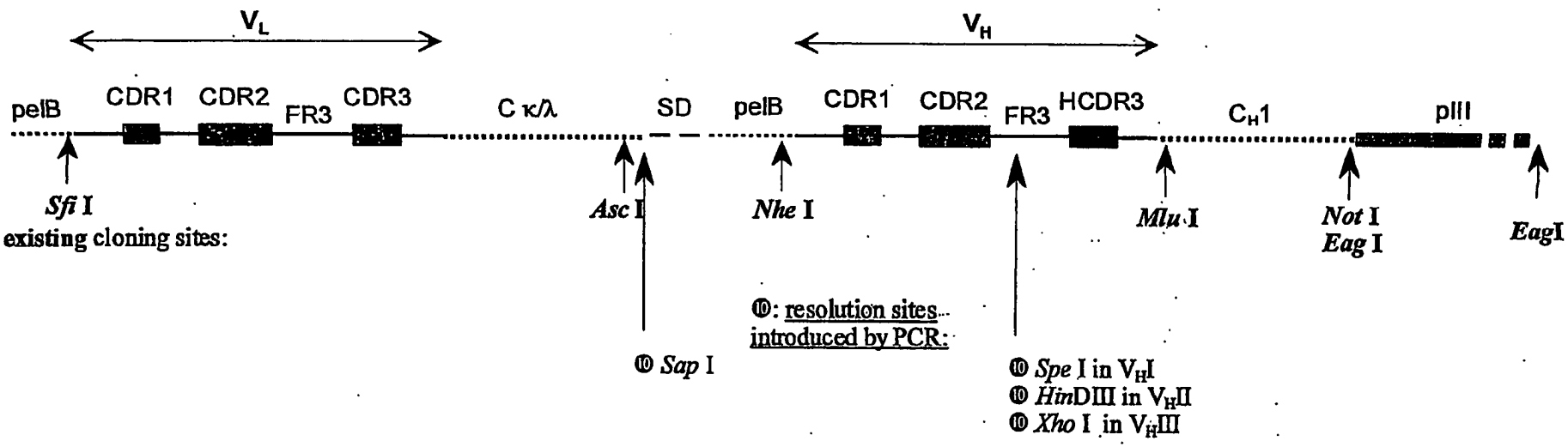


Fig. 2: Expression cassette of huFab vector. CDR: complementarity determining region; C: constant light or constant heavy domain; FR: framework region; pelB: leader sequence; pIII: phage protein pIII; SD: Shine-Dalgarno sequence; V_H: variable heavy domain; V_L: variable light domain

FIG. 2

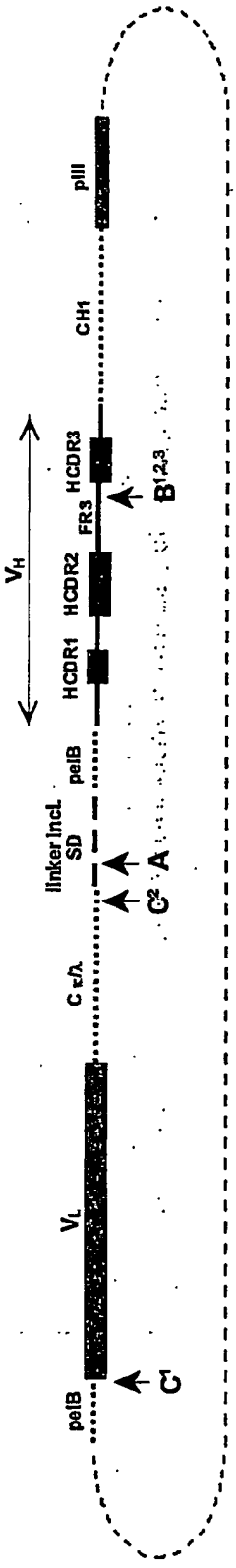


Fig. 3

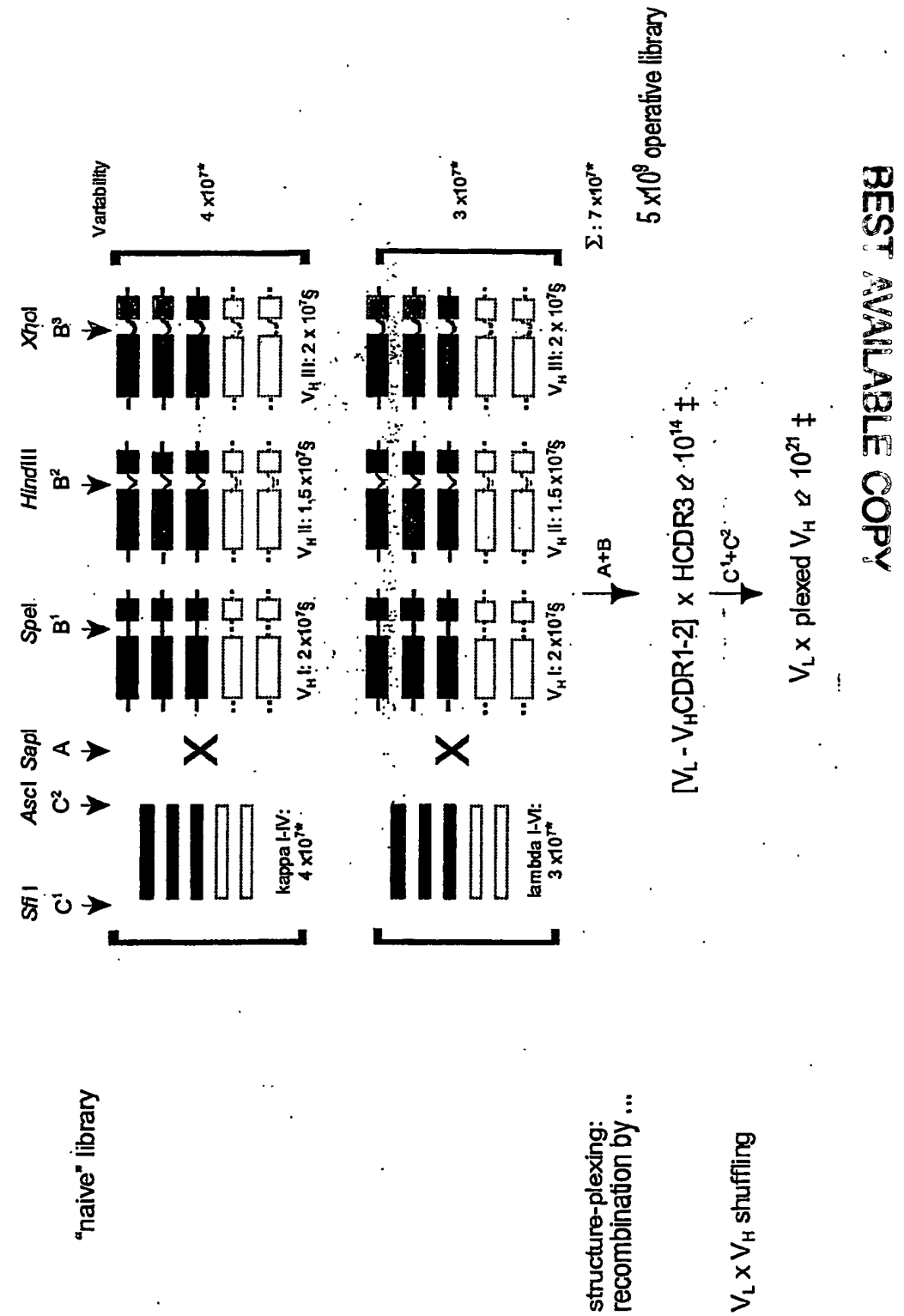


Fig. 4

footnote to figures 3 and 4:
‡ theoretically accessible library size; * real size of unplexed library; § initial complexity of V_H library subsets
For cosmix-plexing® the first restriction enzyme used must generate non-palindromic extensive-ends.
The resolution sites in FR3 of V_H (site B^1, B^2, and B^3 in Fig. 3 and 4) are for three different restriction enzymes (B^1 = *Spe*I for Kabat subgroup V_H1, B^2 = *Hin*dIII for subgroup V_H2; and B^3 = *Xho*I for Kabat subgroup V_H3. This and a characteristic overhang following a *Sap*I restriction at site A between hea and light chain enable conservation of the respective functional framework context.

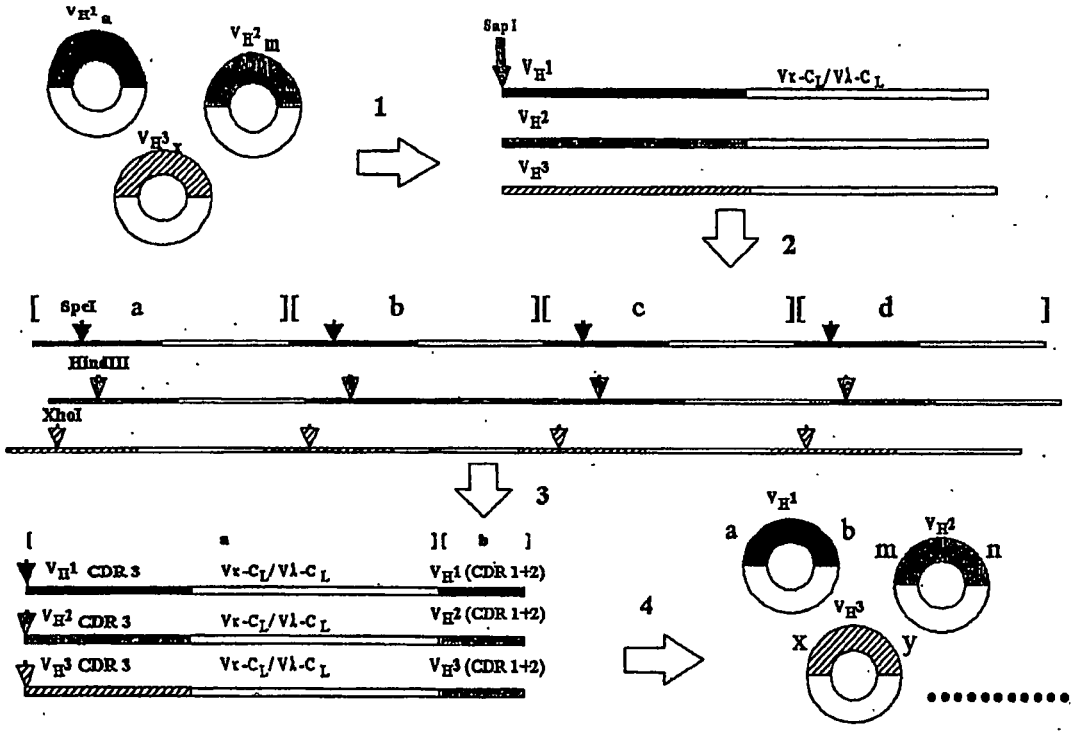


Figure 5

Structure-plexing protocol ensuring high efficiency recombination restricted to within each of the three V_H framework subgroups: In step 1, circular phagemid DNA from the library is cleaved by the type IIs restriction enzyme SapI, which creates a unique non-palindromic cohesive end, which is unique for each VH-framework group. Step 2: When these cleavage products are ligated at high DNA concentration, three sets of concatamers are formed, as shown, each containing only members from the same framework group. Step 3: A second cleavage with the framework group-specific restriction enzymes (SpeI, HindIII and XhoI) leads to resolution of the recombination products, i.e. monomeric units are formed which on ligation at lowe DNA concentration (Step 4) form, by ring closure, phagemid vectors, which have the same general structure as the starting plasmids, but in which the VH CDR3s have been recombined with VH-(CDR1+2) variants from other clones. All these reactions can be carried out within a "one pot" reaction without purification of any fragments. Furthermore, it should be noted that during recombination the VH-CDR3 region remains with the same light chain, a featu which we consider important at early stages when working with large numbers of preselected variants, in order to maintain structural schema. If required entire light and heavy chains can be reshuffled after SfiI and AscI cleavage (not shown in the diagram) with or without the formation of concatamers and their resolution. Variants and recombinants illustrated are shown as single representatives of much larger series

Fig. 6

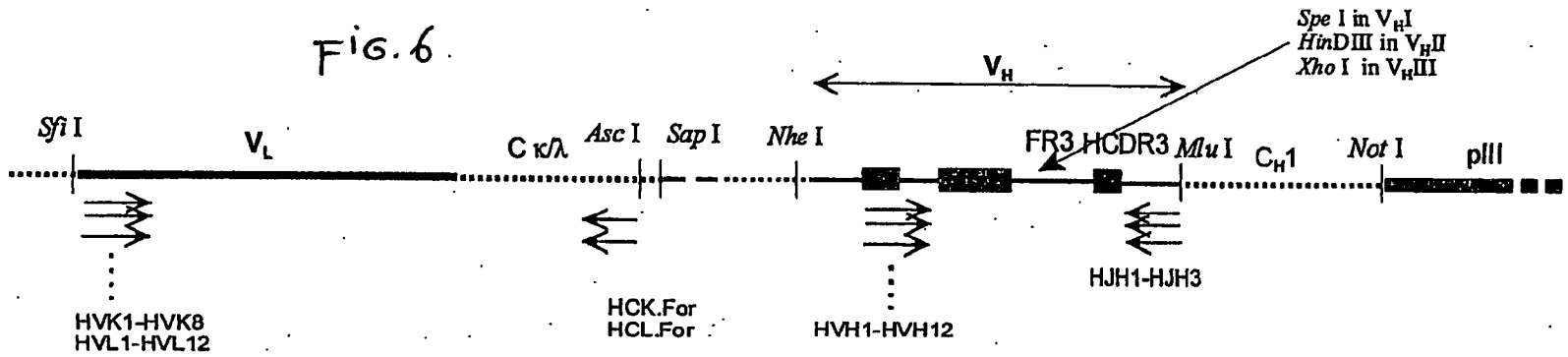


Figure 7

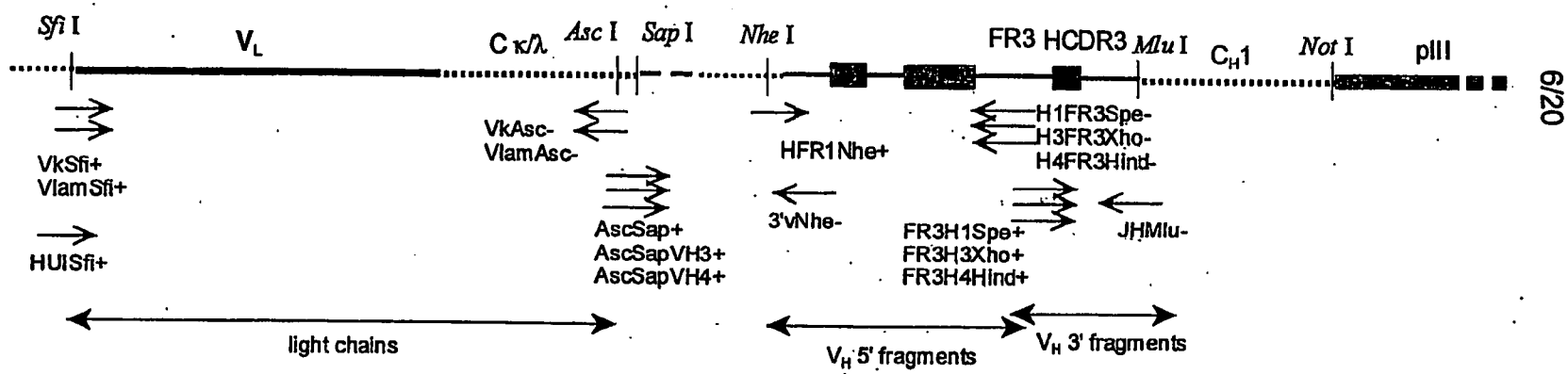


Figure 6: Primers used for the first PCRs of light and heavy chains.

Figure 7: Primers used for the second PCRs of light and heavy chains, introducing the required restriction sites and the VH subgroup-specific *Sap*I sites. *Sap*I was introduced by amplifications using the indicated AscSap-containing sense primers and the non-sense primer 3'vNhe-. The resulting product wa used as mega-primer in an amplification with the primer HUISfiI+.The product was cloned after *Sfi*I and *Nhe*I digestion. Light chain sequences were cloned using *Sfi*I and *Asc*I . Heavy chain fragments were cloned using either *Nhe*I and *Bcu*I, *Hin*dIII, *Xho*I for 5' fragments, or *Mlu*I and *Bcu*I, *Hin*dIII, XhoI for the 3' fragments.

Fig. 8

footnote:
A: Recombination via structure-plexing as in Fig. 3
B: Selection round(s)
Further naive and/or selected clones (1, 2 and/or 3) can be subjected to another round of structure-plexing

Forward and back primer to introduce a restriction site into FR3 of subgroup VHI:

```
          R  V  T  M  T  R  D  T  S  I  S  T  A  Y  M  E  L  S  R  L  R  S  D  D  T  A  V  Y  Y  C  A  R
mostly: AGG GTC ACC ATG ACC AGG GAC ACG TCC ATC AGC ACA GCC TAC ATG GAG CTG AGC AGG CTG AGA TCT GAC GAC ACG GCC GTG TAT TAC TGT GCG AGA
ambig.  CGN GTN ACN ATG ACN CGN GAY ACN WSN ATH WSN ACN GCN TAY ATG GAR YTN WSN CGN YTN CGN WSN GAY GAY ACN GCN GTN TAY TAY TGY GCN CGN
                                    ACT AGT
                                    SpeI
            C ACC ATT ACC GCG GAC ACT AGT TTCCTTCT 3'
        3' g Tgg TAA Tgg CgC CTg TgA TCA AAggAAgA 5'   H1FR3Spe-
```

```
          R  V  T  M. T  R  D  T  S  I  S  T  A  Y  M  E  L  S  R  L  R  S  D  D  T  A  V  Y  Y  C  A  R
mostly: AGG GTC ACC ATG ACC AGG GAC ACG TCC ATC AGC ACA GCC TAC ATG GAG CTG AGC AGG CTG AGA TCT GAC GAC ACG GCC GTG TAT TAC TGT GCG AGA
ambig.  CGN GTN ACN ATG ACN CGN GAY ACN WSN ATH WSN ACN GCN TAY ATG GAR YTN WSN CGN YTN CGN WSN GAY GAY ACN GCN GTN TAY TAY TGY GCN CGN
                                    SpeI
                    5' CCAACCAA ACT AGT ACR AGC ACA GCC TAC ATG G 3'    FR3H1Spe+
```

FIG. 9

Forward and back primer to introduce a restriction site into FR3 of subgroup VHII:

```
          R  V  T  I  S  V  D  K  S  K  N  Q  F  S  L  K  L  S  S  V  T  A  A  D  T  A  V  Y  Y  C  A  R
mostly: CGA GTC ACC ATA TCA GTA GAC AAG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
ambig:  CGN GTN ACN ATH WSN GTN GAY AAR WSN AAR AAY CAR TTY WSN YTN AAR YTN WSN WSN GTN ACN GCN GCN GAY ACN GCN GTN TAY TAY TGY GCN CGN
                                                          AAG CTT
                                                          HinDIII
                                              C CAG TTC TCC CTG AAG CTT TTATTATA 3'
                                            3' g gTC AAg Agg gAC TTC gAA AATAATAT 5'   H4FR3Hind-
```

```
          R  V  T  I  S  V  D  K  S  K  N  Q  F  S  L  K  L  S  S  V  T  A  A  D  T  A  V  Y  Y  C  A  R
mostly: CGA GTC ACC ATA TCA GTA GAC AAG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
ambig:  CGN GTN ACN ATH WSN GTN GAY AAR WSN AAR AAY CAR TTY WSN YTN AAR YTN WSN WSN GTN ACN GCN GCN GAY ACN GCN GTN TAY TAY TGY GCN CGN
                                                          AAG CTT
                                                          HinDIII
                                          5' CCAACCAA AAG CTT AGC TCT GTG ACC GCC GCR 3'   FR3H4HinD+
```

FIG. 9 continued

Forward and back primer to introduce a restriction site into FR3 of subgroup VHIII:

```
          R   F   T   I   S   R   D   N   A   K   N   S   L   Y   L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C   A   R
mostly: CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA
ambig.: CGN TTY ACN ATH WSN CGN GAY AAY GCN AAR AAY WSN YTN TAY YTN CAR ATG AAY WSN YTN CGN GCN GAR GAY ACN GCN GTN TAY TAY TGY GCN CGN
                        CT CGA G
                           XhoI
    ggC CGA TTC ACC ATC TCT CGA GTTATTATA 3'
 3' CCg gCT AAg Tgg TAg AgA gCT CAATAATAT 5'  H3FR3Xho-
```

```
          R   F   T   I   S   R   D   N   A   K   N   S   L   Y   L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C   A   R
mostly: CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA
ambig.  CGN TTY ACN ATH WSN CGN GAY AAY GCN AAR AAY WSN YTN TAY YTN CAR ATG AAY WSN YTN CGN GCN GAR GAY ACN GCN GTN TAY TAY TGY GCN CGN
                        CT CGA G
                           XhoI
              CCAACCAA TCT CGA GAY AAT KCC AAG AAC WC  FR3H3Xho+
```

Fig. 9 continued

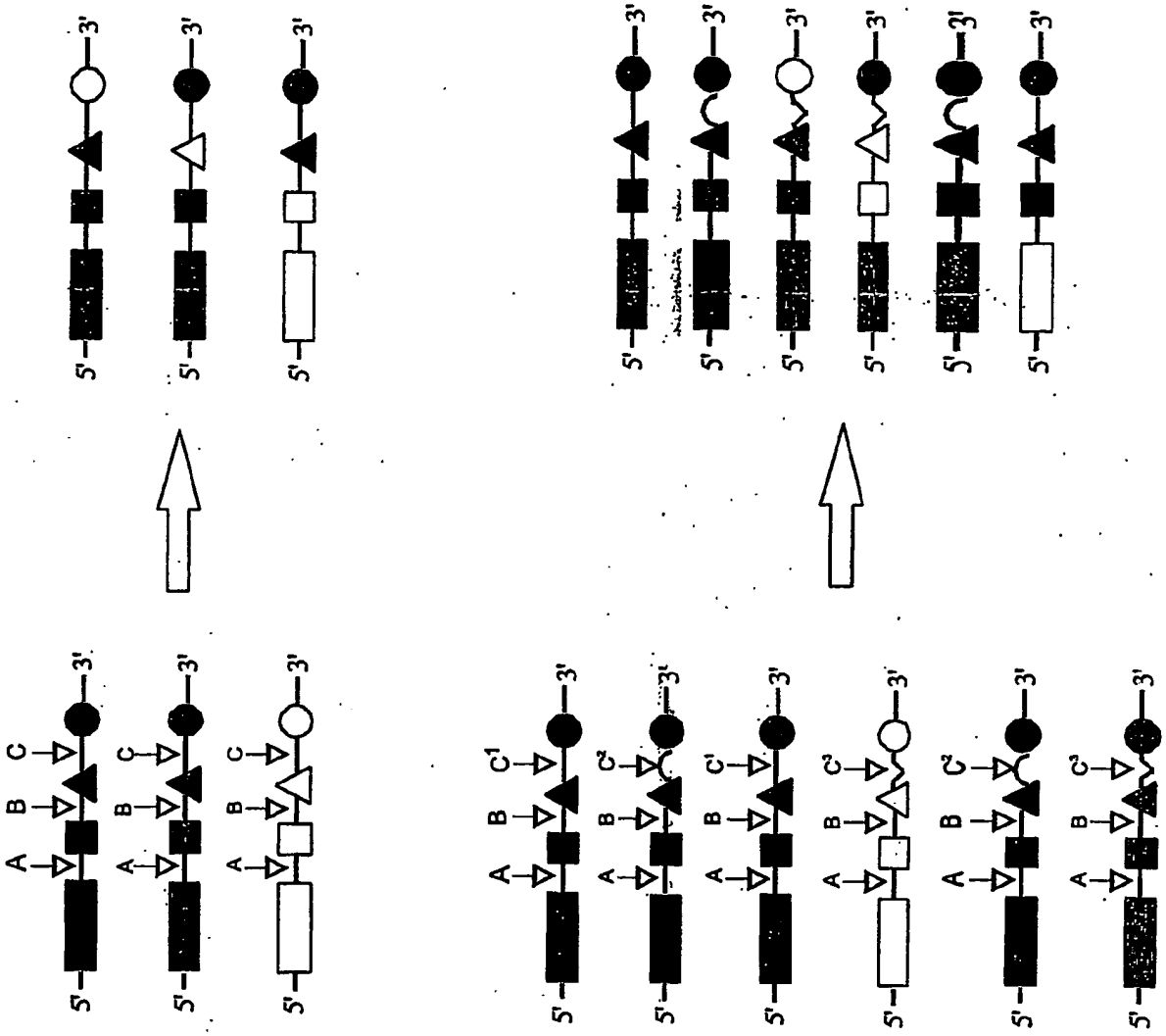


Fig. 10 a

Fig 10 b

Figure 11:

huFab -pro- selections on hVEGF

Numbers of input and resulting phages and the applied conditions of selection are shown.

Panning No.	phage from round	beads	blocker	buffer		phage input	phage output
				incubation	wash / x-times/total time	total	total
1-pro-	naive library	Carboxy	100 mM ethano-lamine	0.001% PBST in 1% fishge-latine	PBST 0.001 % Tween 10x within 35 min	1x 10^{12} cfu	3.8x 10^{4} cfu
structure plexing®: without LC* shuffling: 6.6 x10^{8} new variants; with LC shuffling: 3.3 x10^{8} new variants							
1-pro- after plexing	from plexing	Carboxy	100 mM ethano-lamine	2YT + 0.01 % Tween	PBST 0.05% Tween 12 x within 45 min	2x 10^{11} cfu	2.1 x 10^{6} cfu

* LC: light chain

WO 2004/013328

PCT/EP2003/008305
10/522596

Figure 12:
huFab -pro- selections on hIGF
Numbers of input and resulting phages and the applied conditions of selection are shown.

Panning No.	phage from round	beads	blocker	buffer		phage input	phage output
				incubation	wash / x-times/total time	total	total
1-pro-	naive library	Carboxy	100 mM ethano-lamine	0.001% PBST in 1% fishge-latine	PBST 0.001 % 10x within 35 min	$1x10^{12}$ cfu	7×10^4 cfu
cosmix-plexing®: without LC* shuffling: 3.8×10^8 new variants; with LC shuffling: 5.5×10^7 new variants							
1-pro- after plex-ing	from plex-ing	Carboxy	100 mM ethano-lamine	2YT + 0.01 % Tween	PBST 0.05% Tween 12 x within 45 min	$2x\ 10^{11}$ cfu	$1.5x\ 10^7$ cfu

* LC: light chain

FIG. 13a

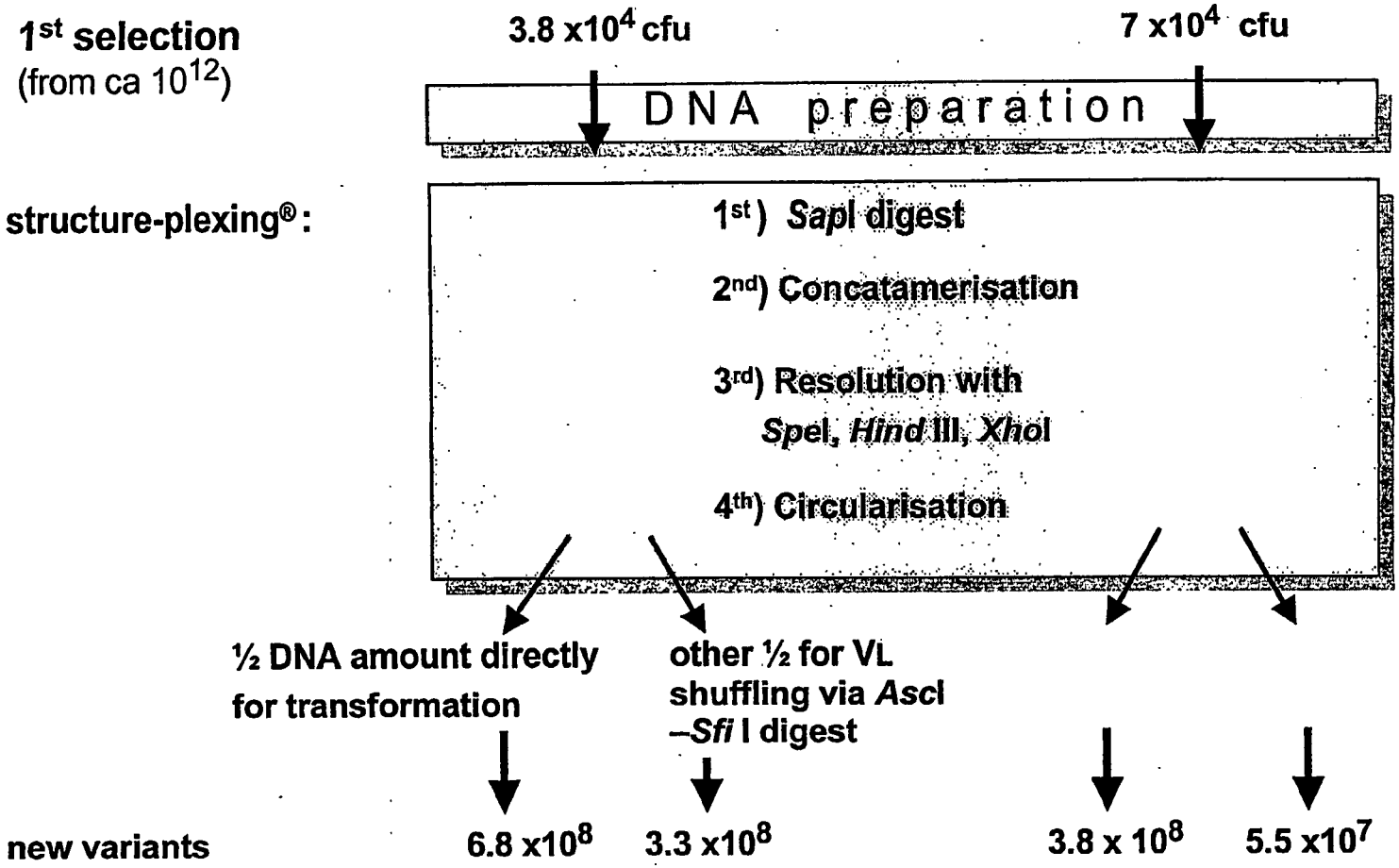


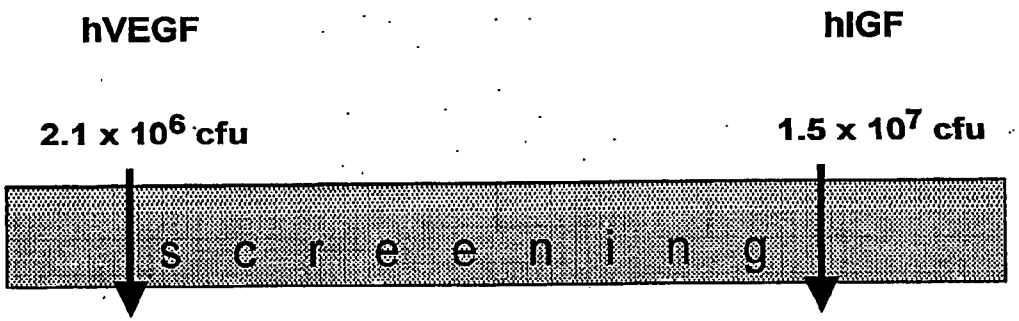
Figure 13b

Phage production from new variants

↓

selection round on respective target:

(challenge with 2 x10^{11} particles)



positives* after selection, structure plexing® + selection:	11	11
positives* after 2 rounds of selection without structure plexing®:	2	9

From 60 chosen randomly: * Positives = Abs. ≥ 0.500 in phage ELISA and ≥ 5fold over background

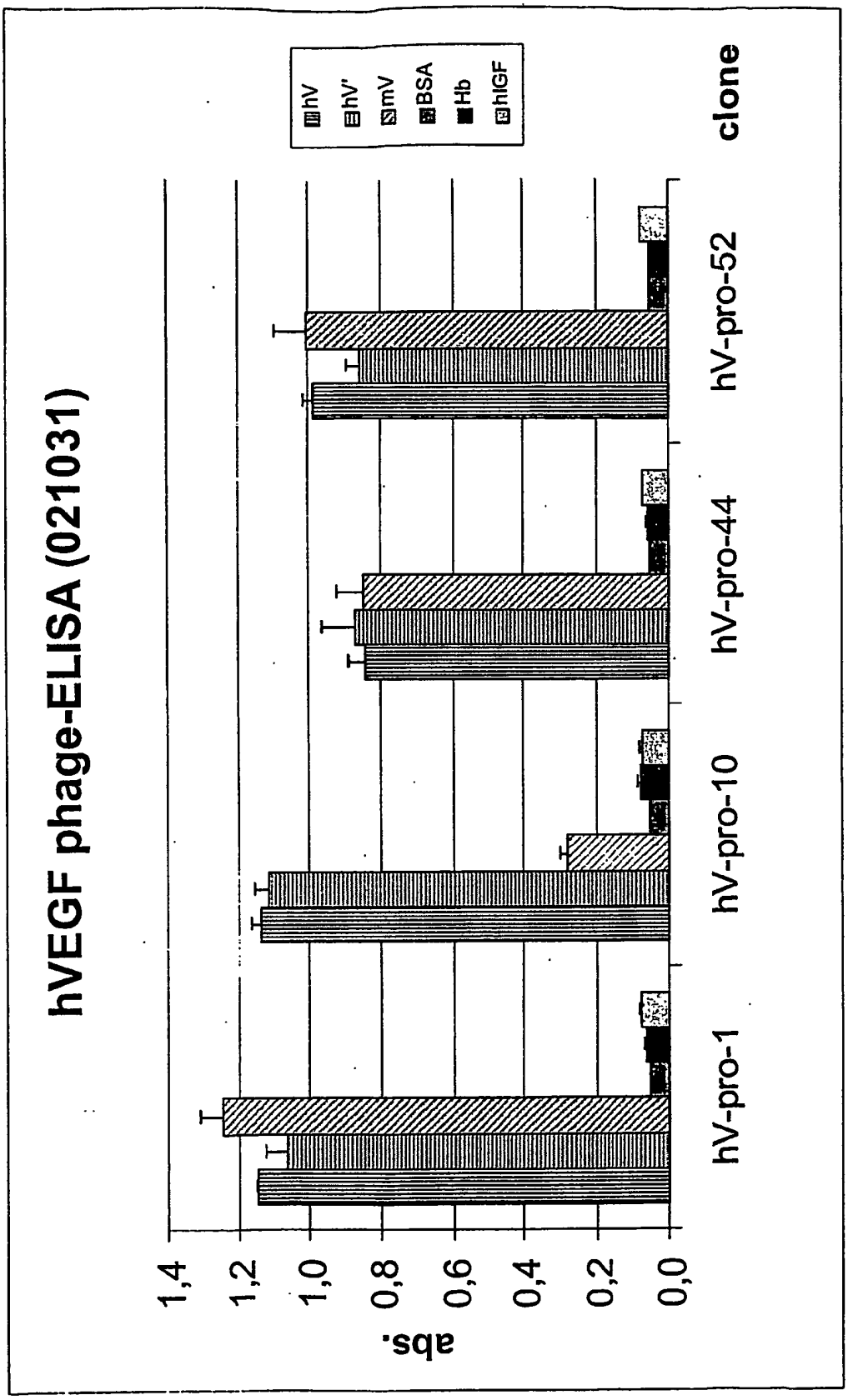


Figure 14

Figure 15

Target: human insulin-like growth factor

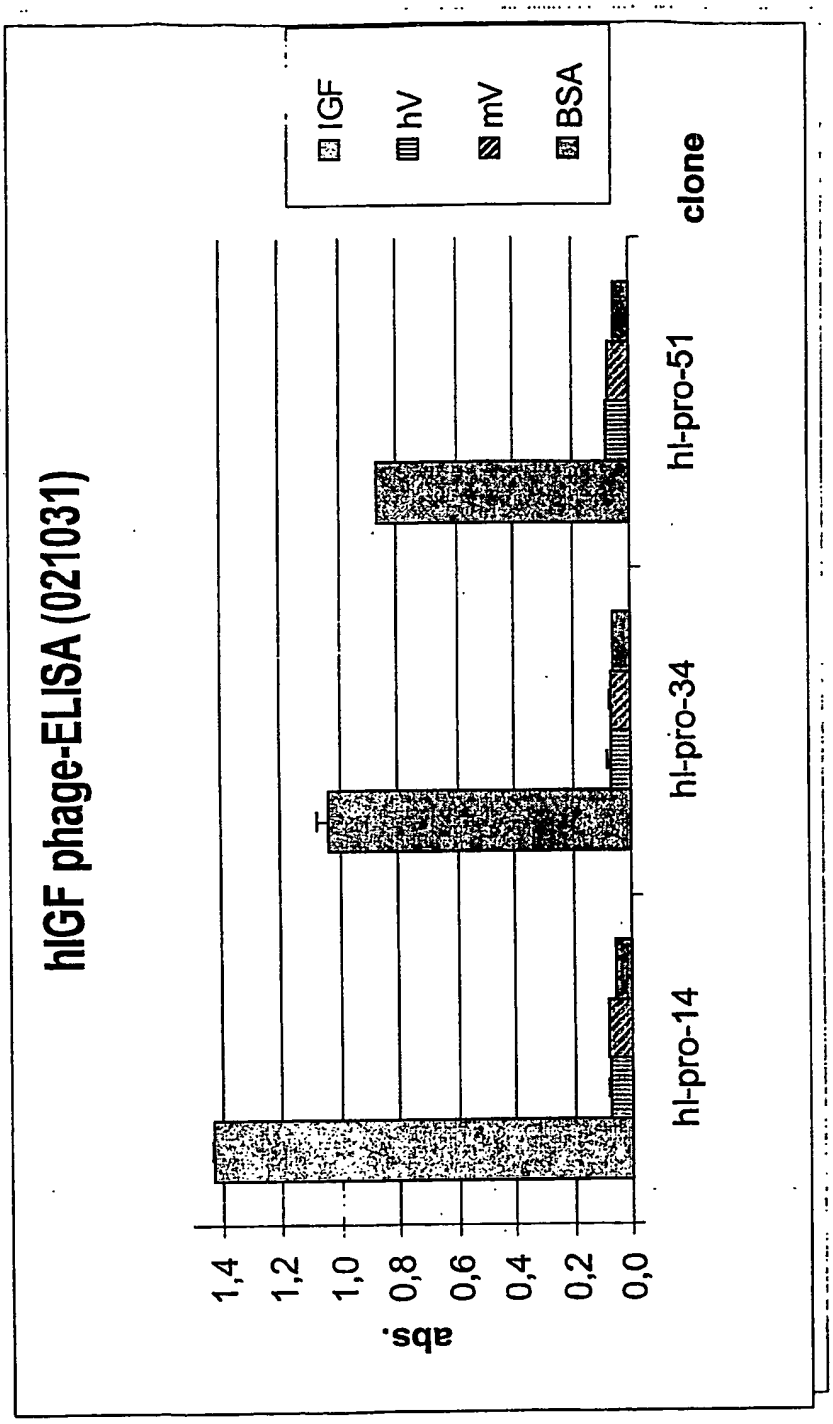
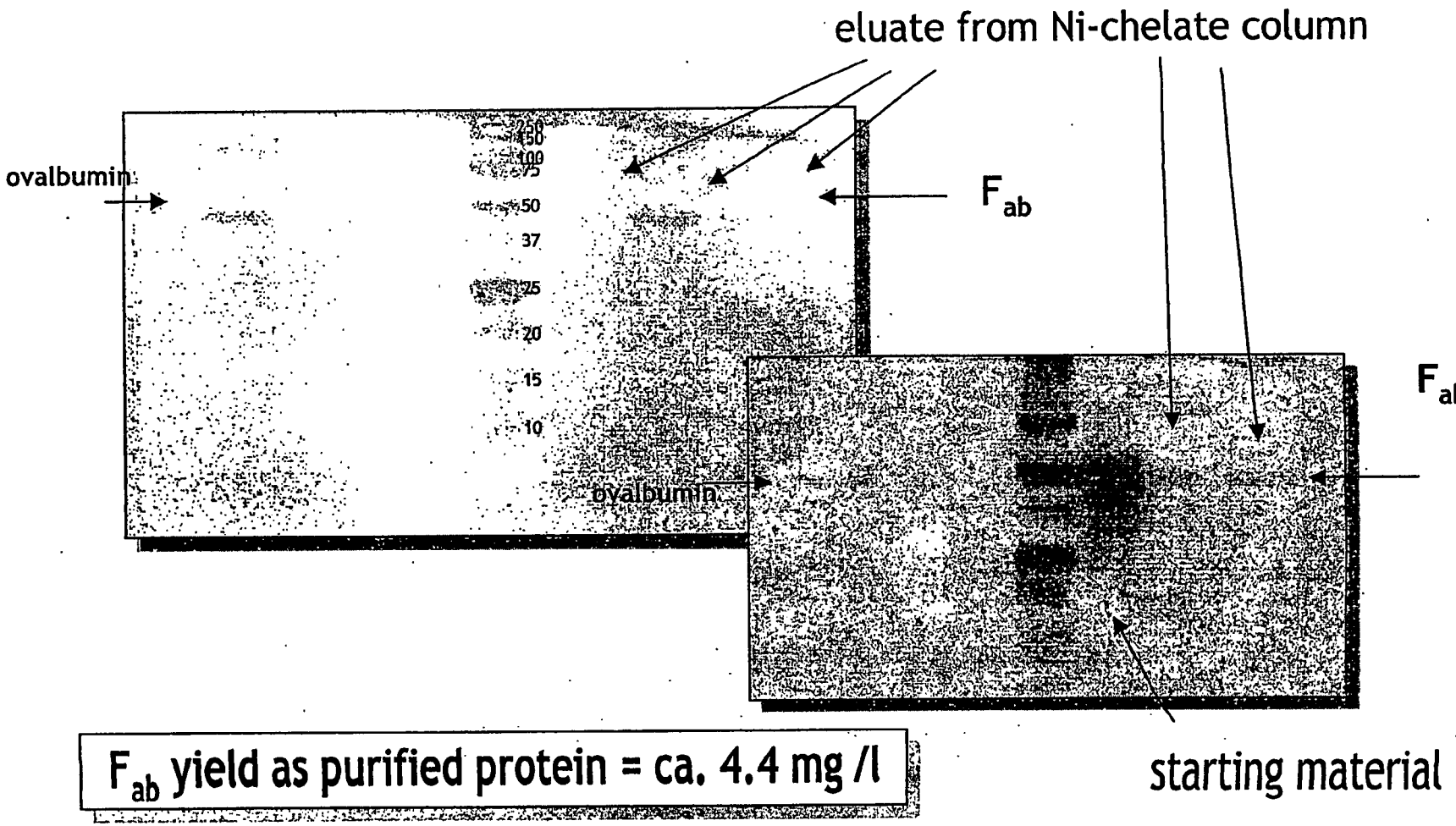


Figure 16

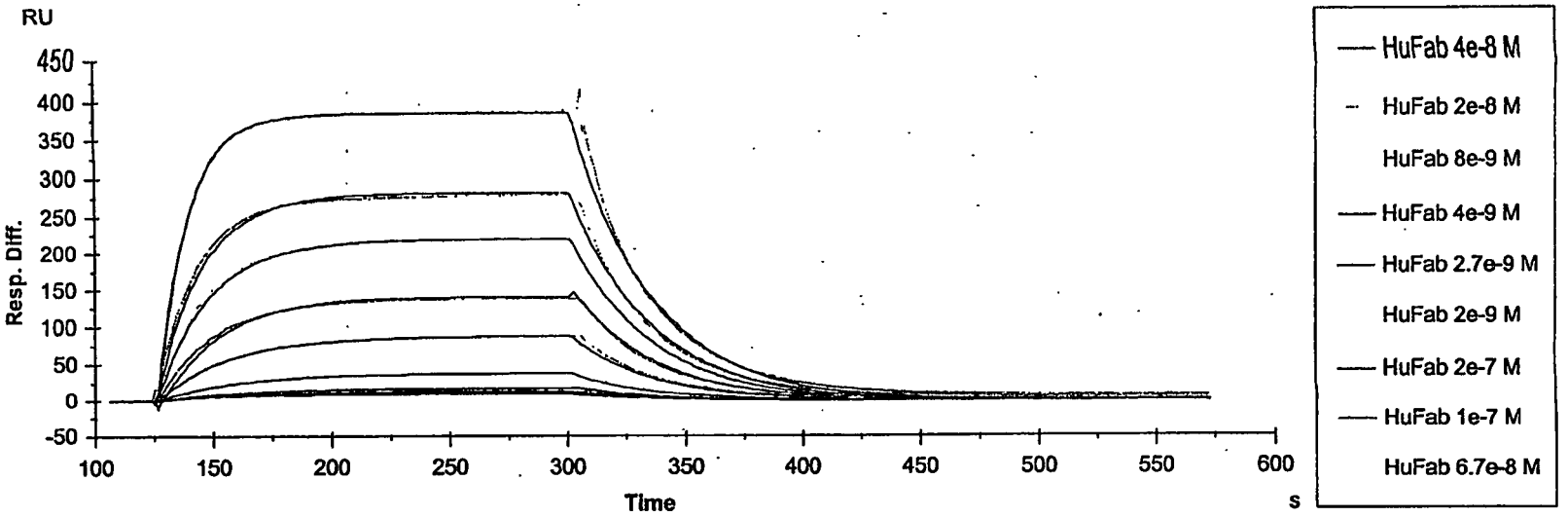
Protein production and purification



F_{ab} yield as purified protein = ca. 4.4 mg /l

Figure 17

Affinity analysis of interaction between huFab and huVEGF



Affinity constants

K_D= 120 nM

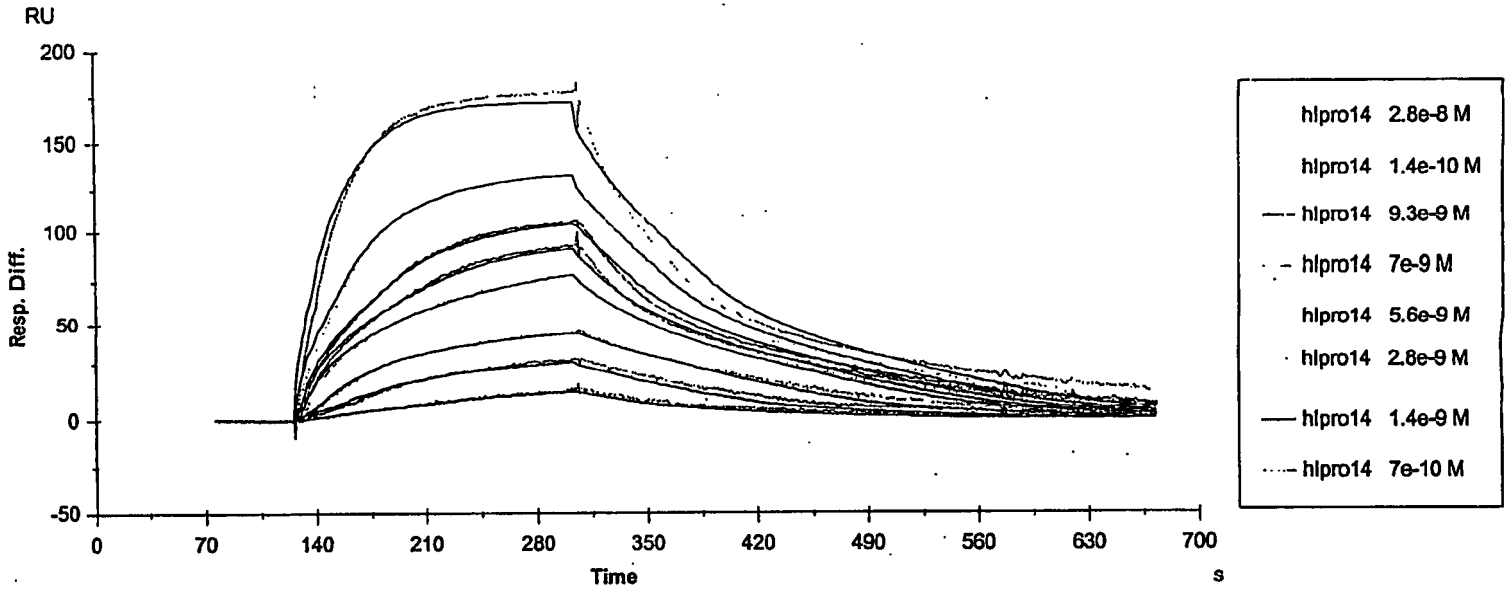
R_{max}= 606 RU, $X^2 = 9$

Rate Equation: 1:1 langmuir binding

$$\frac{dR}{dt} = k_a \times C \times (R_{max} - R) - k_d \times R$$

Figure 18

Affinity analysis of interaction between the huFab protein and hIGF (immobilised)



Affinity constants

K_D= 10 nM

R_{max}= 207 RU, X^2 = 10

Rate Equation: 1:1 langmuir binding

$$\frac{dR}{dt} = k_a \times C \times (R_{max} - R) - k_d \times R$$